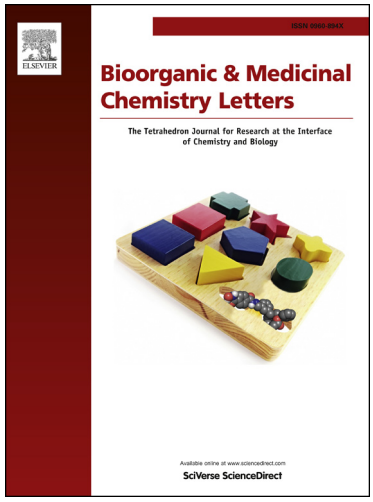

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Highly potent and selective pyrazolylpyrimidines as Syk kinase inhibitors

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Keywords: Spleen tyrosine kinase; Pyrazolylpyrimidine; Syk inhibitors; Rheumatoid arthritis; Structure-activity relationship

ABSTRACT

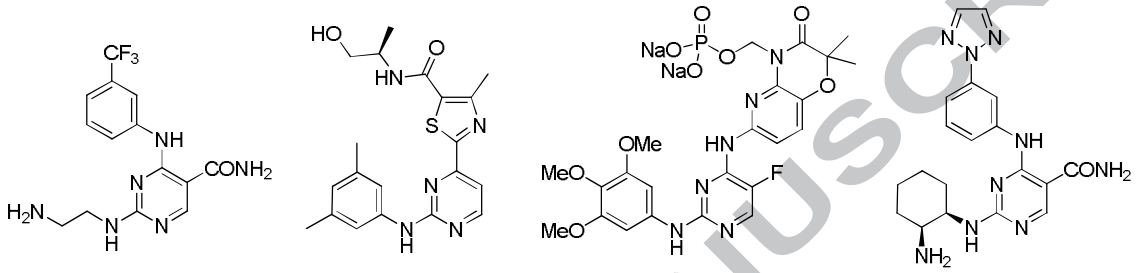
A series of pyrazolylpyrimidine scaffold based Syk inhibitors were synthesized and evaluated for their biological activities and selectivity. Lead optimization efforts provided compounds with potent Syk inhibition in both enzymatic and TNF-α release assay.

Spleen tyrosine kinase (Syk) is an intracellular non-receptor protein tyrosine kinase that is involved in immunoreceptor signaling events, mainly in B-cell receptor, Fc receptors, integrin and lectin receptors.[1] Syk is most widely expressed in hematopoietic cells like B cells, mast cells, macrophages and neutrophils but is also expressed in other tissues.[2]

Upon engagement with these receptors Syk performs an important function in signal transduction which mediates diverse cellular responses, including proliferation, differentiation and phagocytosis.[3] Dysregulation and/or misregulation of different signal transduction pathways of Syk in different cell types have been implicated in numerous diseases and disorders e.g., allergic rhinitis, asthma, autoimmune diseases, rheumatoid arthritis (RA), osteopenia, osteoporosis, COPD and various leukemia and lymphomas.[4-8] Thus, the inhibition of Syk activity may offer a therapeutic option for treatment of many diseases associated with Syk activity.

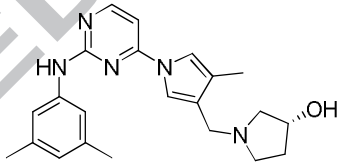
Over the last few years a number of ATP-competitive Syk inhibitors of varied chemotypes have been described[9] and it has been reported that pharmacological inhibition of Syk kinase activity modulates mast cell degranulation, leukocyte immune function and suppress inflammation *in vivo*.[10,11] Aminopyrimidine core scaffold is well recognized in discovery of kinase inhibitor as it provides an essential hinge binding motif. Several aminopyrimidine based Syk inhibitors are know in the literature. Few examples are Astellas compound **1**[12] , Vertex's compound **2**[13], Rigel's compound **3** (R-788), and Portola's compound **4**[14] (PRT062607). Clinical development of R-788 for rheumatoid arthritis was halted due to limited efficacy in patients inactive to biologic

TNF inhibitors.[15] Other few molecules are in early phases of clinical studies, including R-343 for asthma[16] and PRT062607 for autoimmune diseases. PRT062607 (also known as BIIB-057, **4**) was the only selective Syk inhibitor that has been evaluated in clinical studies. However, a planned phase II trial in rheumatoid arthritis (RA) was withdrawn prior to patient enrollment. Recently, Syk inhibitors with different scaffolds were also reported. Among them are thiazolopyrimidine and triazolopyridine derivative from Hoffmann-La Roche[17], triazine amide compounds from Novartis[18], and phenyl carboxamide compounds from Merck.[19] These compounds were discontinued partly due to issues related to cellular permeability, solubility and or toxicity.



1 **2** **3** **4**

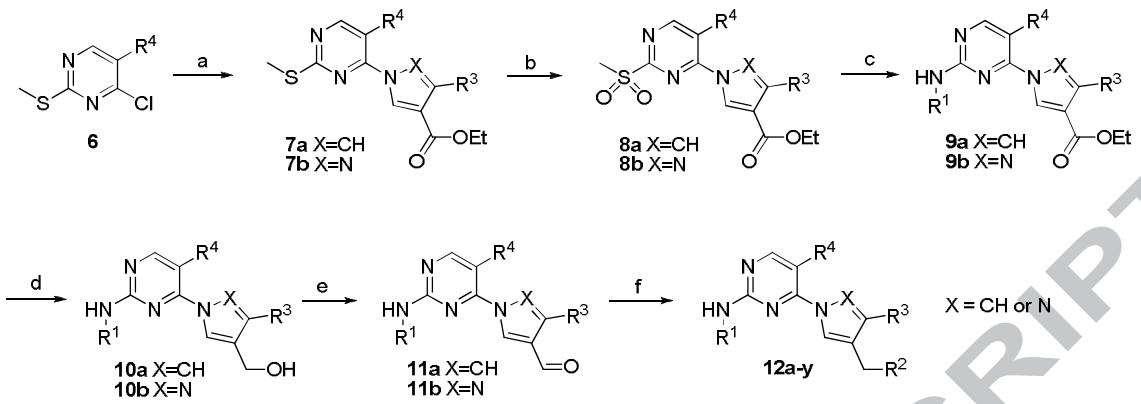
In our efforts to discover novel, potent and selective Syk inhibitors with improved pharmacological profiles, cheminfomatic analysis and high throughput screening of in-house focused chemical library of pyrimidine scaffold were carried out. Pyrrolylpyrimidine derivative **5**[20] was identified as a potent Syk inhibitor and was selected for further optimization.



5 IC_{50} = 2.3 nM

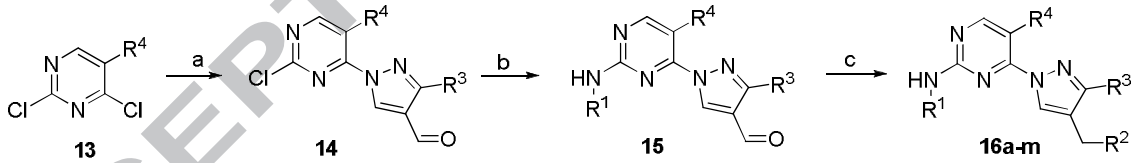
Herein, we wish to report our findings on pyrrolylpyrimidine and pyrazolylpyrimidine derivatives as potent and selective Syk inhibitors with good pharmacological profiles.

Schemes 1 & 2 outline the general synthetic procedures[20, 21] for the final arylaminopyrimidine compounds. The pyrrolylpyrimidine and pyrazolylpyrimidine derivatives **12a-y** were synthesized starting from 4-chloro-2-(methylthio)pyrimidine **6**. The coupling reaction of pyrimidine **6** with pyrrole or pyrazole esters gave pyrrolyl- and pyrazolylpyrimidine esters **7**. Activation of the sulfide group of the compounds **7** via oxidation with mCPBA to sulfones **8**, followed by displacement of methanesulfonyl group with a variety of arylamines afforded a series of 2-arylaminopyrimidine **9**. Subsequent reduction of the esters **9** using lithium aluminumhydride gave the intermediate alcohols **10**, which were further oxidized with PDC to afford pyrrole- and pyrazole aldehydes **11**. Finally, the desired compounds **12a-y** were successfully obtained from reductive amination of pyrrole- and pyrazole aldehydes **11** with appropriate amines.



Scheme 1. Reagent and conditions: (a) pyrrole (pyrazole) ester, K_2CO_3, CH_3CN, reflux, 80%; (b) mCPBA, CH_2Cl_2, rt, 83%; (c) R^1NH_2, c-HCl, EtOH, reflux, 30-50%; (d) $LiAlH_4$, THF, 0°C, 90%; (e) PDC, CH_2Cl_2, rt, 90%; (f) R^2NH, $NaBH(OAc)_3$, Et_3N, CH_2Cl_2, rt, 70-90%

Similarly, 2-indolylamino substituted pyrazolylpyrimidine derivatives **16a-m** were prepared according to the synthetic method shown in Scheme 2. Compounds of general structure **16a-m** were synthesized in three-step procedures starting from 2,4-dichloropyrimidine **13**. The compound **13** was reacted with pyrazole aldehyde[22] in acetonitrile to give pyrimidine pyrazole aldehyde derivative **14** as a mixture with 2-substituted and 2,4-disubstituted compounds. Then, the desired 4-substituted isomer **14** was isolated as a single regioisomer by a silica gel column chromatography and used in the following reaction. Further coupling reaction of the compound **14** with various aniline derivatives via a Buchwald-Hartwig condition or acid catalyzed condition afforded 2-anilino pyrimidine pyrazole aldehyde derivative **15**. Finally, the desired compounds **16a-m** were prepared from the compound **15** by reductive amination with appropriate amines.



Scheme 2. Reagent and conditions: (a) pyrazole aldehyde, K_2CO_3, CH_3CN, reflux, 40-45%; (b) R^1NH_2, $Pd(OAc)_2$, Xantphos, K_2CO_3, dioxane, reflux or R^1NH_2, c-HCl, EtOH, reflux, 40-50% ; (c) R^2NH, $NaBH(OAc)_3$, TEA, CH_2Cl_2, rt, 60-80%

Compounds **12a-y** and **16a-m** were tested for inhibitory activity against Syk kinase and selectivity against a small panel of other key kinases. Kinase screening activity revealed that 3,5-disubstituted aniline is optimal for Syk inhibitory activity. Syk inhibitory activity data of 2-aniline substituted pyrimidine derivatives with a concomitant substitution of various aminomethyl groups at pyrrole or pyrazole moiety are shown in Table 1. Compound **5**, 3,5-dimethylanilino compound with substitution on 4 position with aminopyrrole, showed potent Syk inhibitory activity with an IC_{50} value of 2.3 nM. The first modification was focused on introducing different

heteroaryl group as a linker and different amine group instead of pyrrole linker to improve its selectivity and potency. Pyrrole or pyrazole linker with 4-5 membered cyclic aminomethyl substituted compounds (**5**, **12a**, **12c** and **12d**) showed generally high inhibitory activity against Syk. However, Syk inhibitory activity was not improved when a bigger 6-membered cyclic amine was employed (compound **12g**). Interestingly, 4,4-difluoropiperidine analogue **12h** was about 133-fold less active than compound **5**. The decreased activity of 4,4-difluoropiperidine derivative **12h** might be due to the lowered basicity of tertiary amine by strong electron-withdrawing difluoro substitution. Acyclic amino groups attached compounds were also investigated. *N,N*-dimethylamine derivative **12e** strongly inhibited Syk similar to cyclic amine derivatives. Second modification performed on 2-position of pyrimidine. The replacement of 3,5-dimethylaniline by trimethoxyaniline such as in compound **12b** and **12f** resulted in a 7-fold and 12-fold decrease in activity against Syk, respectively. Derivatives **12i** and **12j** with cyclopropyl substituent in R^3 position of pyrrole ring showed similar Syk inhibitory activity compared to corresponding methyl analogs **5** and **12a**. Interestingly, replacing the hydroxyl group in pyrrolidine or azetidine with an amino or carboxyl group (compounds **12K** and **12l)** showed modest decrease in Syk inhibitory activity in comparison to compound **5**.

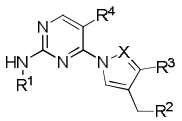
Modifications at 4'-position of aniline portion of the compound **5** was undertaken in order to improve physicochemical properties and activity and the data is presented in Table 2. Substitution of hydroxyethyloxy group to 4'-position of the compound **5** leaded to the compound **12n** of which kinetic solubility (25 mM DMSO stock) was increased to 120 µM compared to 70 µM of the compound **5**. Cell permeability of the compound **12n** in Caco-2 cell was also increased with Papp of 10.3 x 10^{-6} cm/s compared to **5** with Papp of 2.0 x 10^{-6} cm/s. Inhibitory activity of the compound **12n** against CYP 3A4 isozyme was improved with IC_{50} value of >10 µM compared to the compound 5 with IC_{50} value of 5.1 µM. All these improvement are partly dues to a decrease in lipophilicity based on the comparison of the calculated cLogP values of **12n** (3.64) and **5** (4.59) using ChemBioDraw software. Changes at 4'-position of aniline with hydroxyethyloxy, pyrrolidinylamidomethoxy, pyrrolidinylethoxy, methoxy and methanesulfonate, **12m** (IC_{50} 5.0 nM), **12o** (IC_{50} 7.3 nM), **12s** (IC_{50} 5.7 nM), **12t** (IC_{50} 6.3 nM), respectively, had lower Syk inhibitory activities. Azetidinol substituted derivatives, **12m**, **12o** were 2-3.5 fold more potent than pyrrolidinol derivatives. C4 position with heterocycle (pyrrole or pyraz ole) on pyrimidine ring had similar activities (**12r** vs **12q**). It was interesting to see that 3,5-dimethoxy aniline derivatives, **12v**, **12w** were 7-8 fold less active than 3,5-dimethyl aniline substituted derivative, **12o**, **12p**. Moreover, larger group like 4'-phenoxy substituted compounds **12x** (IC_{50} 144.5 nM), **12y** (IC_{50} 377 nM) lost its inhibitory activity significantly.

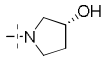
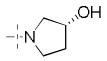
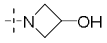
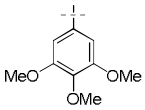
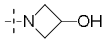
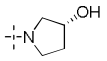
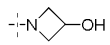
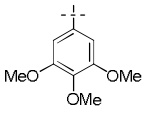
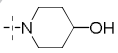
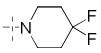
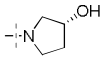
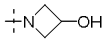
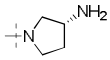
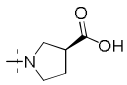
Further elaboration of C2-substitution at pyrimidine scaffold by employing indoline, indole and indazole was undertaken. Derivatives of compound **16** were similarly prepared from corresponding aminoindoline, aminoindole and aminoindazole following Scheme 1 or 2 above. Syk inhibitory activity data of compound **16** derivatives are shown in Table 3. There was not much difference in activity by varying N-alkyl substituent at indole ring (**16b** and **16d**). Cyclopropyl variant at R^3 position of compound **16c** showed about 2.5-4 fold increase in activity (**16b** and **16d**). Modification on C5 position of pyrimidine with flourine resulted in dramatic loss of inhibitory activity as seen in compound **16l** compared to non-substituted derivative **16i**. Further

derivatization with 4-fluoropyrimidine compound was not carried out. Surprisingly 3'-chloro substitution at indole or indazole ring (**16e**-**16g** and **16k**) showed excellent Syk inhibitory activity with IC_{50} values of 0.7 nM, 1.0 nM, 1.0 nM and 0.7 nM, respectively. These compounds (**16f** and **16g**) also showed high cellular activity in THP-1 cell with IC_{50}'s of 38 nM and 44 nM, respectively (Table 4). However, similar electron withdrawing CN substitution at 3'-position of indole (**16m**) was not highly active partly due to hydrophilic nature of CN compared to chlorine atom.

Table 1

Syk inhibitory activity[23] of compounds **5** and **12a-12l**

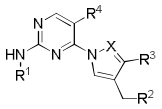


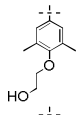
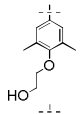
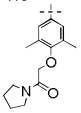
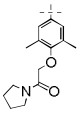
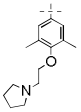
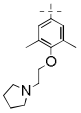
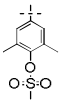
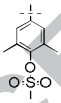
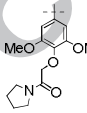
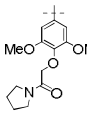
Compound	R^1	R^2	R^3	R^4	X	Syk IC_{50}[a] (nM)
5			CH_3	H	CH	2.3
5a			CH_3	H	N	871
12a			CH_3	H	CH	5.4
12b			CH_3	H	CH	38.0
12c			CH_3	H	N	3.5
12d			CH_3	H	N	7.3
12e			CH_3	H	CH	6.2
12f			CH_3	H	CH	71.8
12g			CH_3	H	N	39.1
12h			CH_3	H	CH	307
12i				H	CH	3.7
12j				H	CH	1.9
12k			CH_3	H	CH	13.2
12l			CH_3	H	CH	17.8

[a] IC_{50} values are averages of two or three experiments.

Table 2

Syk inhibitory activity of compounds **12m-12y**

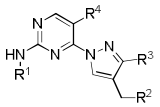


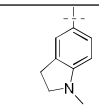
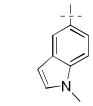
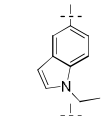
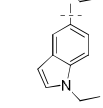
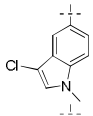
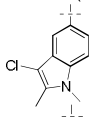
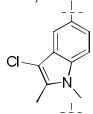
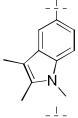
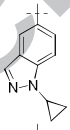
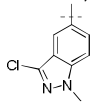
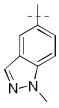
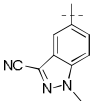
Compound	R^1	R^2	R^3	R^4	X	Syk IC_{50}[a] (nM)
12m			CH_3	H	CH	5.0
12n			CH_3	H	CH	17.3
12o			CH_3	H	CH	7.3
12p			CH_3	H	CH	14.4
12q			CH_3	H	CH	11.5
12r			CH_3	H	N	8.9
12s			CH_3	H	CH	5.7
12t			CH_3	H	CH	6.3
12u			CH_3	H	CH	7.9
12v			CH_3	H	CH	61.4
12w			CH_3	H	CH	100.8
12x			CH_3	H	CH	144.5
12y			CH_3	H	CH	377

[a] IC_{50} values are averages of two or three experiments.

Table 3

Syk inhibitory activity of compounds **16a-16m**



Compound	R^1	R^2	R^3	R^4	Syk IC_{50}[a] (nM)
16a			CH_3	H	20.4
16b			CH_3	H	36.6
16c				H	9.2
16d			CH_3	H	22.8
16e			CH_3	H	0.7
16f			CH_3	H	1.0
16g			CH_3	H	1.0
16h			CH_3	H	1.7
16i			CH_3	H	19.5
16j			CH_3	H	8.0
16k			CH_3	H	0.7
16l			CH_3	F	326
16m			CH_3	H	5994

[a] IC_{50} values are averages of two or three experiments.

To evaluate the kinase selectivities compounds **16f**, **16g** and **16h** kinase profiling was performed against an extended panel of tyrosine and serine/threonine kinases (Table 4). Within the protein tyrosine kinase (PTK) group, **16f**, **16g** and **16h** was selective (>50-fold) over representatives from the ZAP-70, JAK2, JAK3, Aurora B, KDR and RET families. Compound **16f** was further tested in a kinase selectivity panel (298 kinases at 0.1 and 0.01 μM concentration), and the kinases most potently inhibited were Syk (100 and 96%, respectively) and MLK1 (91 and 44%, respectively).[24]

Table 4

In vitro selectivity of Syk inhibition[23, 25] over other tyrosine kinases

Compound	IC_{50} (μM)	IC_{50} (nM)						
	THP-1	SYK	ZAP-70	JAK2	JAK3	Aurora B	KDR	RET
5	0.077	2.3	NT	115.3	349.1	NT	NT	NT
16f	0.038	1.0	53.85	111.9	78.12	1711	191.9	67.8
16g	0.044	1.0	73.42	162.4	478.1	NT	NT	9594
16h	0.056	1.7	995.8	138.2	418.2	985.2	653	NT

NT: not tested

In order to understand the binding mode of our Syk inhibitor X-ray co-crystal structure[26] of compound **16h** bound to the kinase domain of Syk was solved (Fig. 1). From the crystal structure, pyrimidine N1 nitrogen of the compound **16h** serves as a hydrogen bond acceptor for the amine nitrogen of Ala451 in the hinge region and aniline nitrogen forms hydrogen bond with Ala451 carbonyl oxygen. Tertiary amine nitrogen of azetidinol ring forms a hydrogen bond with Asp512 carbonyl oxygen as ammonium ion type. Hydroxyl group in azetidinol forms another hydrogen bond with the carbonyl group of Arg498. In addition to hydrogen bondings compound **16h** was confirmed to have many hydrophobic interactions with the amino acid residues around the binding site. First, the indole ring is exposed to the existing Gly454, Pro455 and the solvent region, and a pyrimidine makes hydrophobic interactions with each of Ala400 and Leu501. The pyrazole ring moiety and azetidinol have interaction with Val385 and Asp512, respectively. Therefore, our Syk inhibitors are expected to show good activity and selectivity for Syk based on these interactions with kinase domain of Syk.

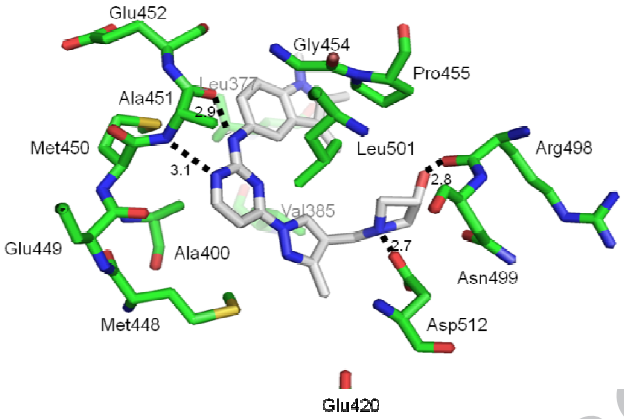


Figure 1. X-ray co-crystal structure of the Syk kinase domain with compound **16h** (PDB code 4RSS).

Pharmacokinetic studies were performed for the highly active and selective compounds in order to evaluate pharmaceutical profiles. Compounds **16f**, **16g** and **16h** have good human liver microsomal stabilities (>60 min, respectively) and good Caco-2 cell permeability (Papp in (cm/s) 10^{-6} **16f** AB: 2.7, BA: 3.8; **16g** AB: 6.2, BA: 7.3; **16h** AB: 6.2, BA: 9.9). These compound showed >10 μM of IC_{50} values for five CYP isozymes. These compounds demonstrated high human plasma protein binding (% free fraction, <0.4%). Compound **16f** (IC_{50} 24.2 μM) and **16h** (IC_{50} 12.5 μM) have moderate hERG inhibition[27] up to 10 μM and also negative in an Ames assay.[28] *In vivo* pharmacokinetic studies for the most potent 3,5-dimethyl aniline compound (**5**) and 4-alkoxy-substituted aniline compound (**12s**) showed poorly bioavailability (F = 7.6% and 7.8%, respectively) in rats. However, C-2 indole and indazole substituted compounds (**16f** and **16h**) had good pharmacokinetic properties with moderate and high bioavailabilites (F = 28.4% and 61%, respectively) (Table 5). The compound **16f** also had high bioavailability of 46.4% *in vivo* PK studies in dogs (Table 6). Thus, compound **16f** was selected as the lead compound for further development.

Table 5

Pharmacokinetic parameters for **5**, **12s**, **16f** and **16h** in male Sprague-Dawley rats (iv 10 mg/kg; po 10 mg/kg), where values are means of n = 3

Compound	CL[c] (mL/min/kg)	V_{ss}[d] (L/kg)	$T_{1/2}$[e] (h)	F[f] (%)	AUC_{last}[g](ng h/mL)
5[a]	43	4.3	2.6	7.6	6240
12s[a]	70	5.6	1.2	7.8	550
16f[b]	26	3.3	3.6	28.4	3233
16h[b]	21	3.7	3.4	61	4000

[a] PEG400/Saline/DMSO (40:55:5)

[b] 20% SBECD + 0.5% acetic acid, iv 5mg/kg.

[c] Clearance.

[d] Volume of distribution at steady state

[e] Terminal half life.

[f] Bioavailability.

[g] Area under curve from time of dosing to last observation

Table 6

Pharmacokinetic parameters of compound **16f** in dog[a]

Compound	**16f**	
Route	IV[b]	PO[b]
Dose (mg/kg)	5	10
CL (mL/min/kg)	40.4	_
V_{ss} (L/kg)	7.1	_
$T_{1/2}$ (h)	4.6	_
AUC_{last} (ng h/mL)	2093	1941
F (%)	_	46.4

[a] Each value is an average of data from three animals.

[b] 20% HPBCD in saline

Evaluation of *in vivo* efficacy of the compound **16f** was studied in a mouse Collagen Induced Arthritis (CIA) model. The model mice were treated orally with 10 and 30 mg/kg of the compound **16f** after arthritis induction for 3 weeks. The compound **16f** showed a significant reduction in the average clinical score with 30% and 50% respectively at doses of 10 and 30 mg/kg.[29] From these studies it can be safely concluded that **16f** significantly reduced the severity and development of arthritis.

A series of novel pyrazolylpyrimidine derivatives as Syk inhibitors have been reported. The strong Syk inhibitory activity was achieved when the 2 position of the pyrimidine ring was substituted with 3,5-dimethyl aniline and 4-alkoxy-substituted aniline (**12a-y**). These compounds, however, had poor pharmacokinetic properties. To improve drug like properties, indole or indazole amine was introduced at 2 position on the pyrimidine ring. Compounds (**16a-m**) gave the better activities and improved pharmacokinetic properties. On the basis of the SAR studies of indole and indazole amine substituted derivatives we have optimized the structures and finally identified **16f** as the most potent Syk inhibitor with desirable pharmaceutical profiles. Compound **16f** had excellent Syk inhibitory activity with IC_{50} of 1.0 nM and an oral bioavailability of 46.4% in dogs. Based on these results it was decided to develop compound **16f** for treatment of autoimmune diseases such as rheumatoid diseases.

Acknowledgment

This work was supported by the Korea Drug Development Fund (KDDF) grant funded by the Korea government (MSIP, MOTIE, MW) (No. KDDF2011-06) and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2011-0018355).

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21. Compounds **16a-m** were prepared according to procedures described in PCT Int. Appl. WO 2013109882A1, 2013.

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23. *Cell free kinase assay*: Compounds of the invention were initially diluted to 10mM in 100% DMSO (CALBIOCHEM) for storage and made into kinase buffer solution to create a compound concentration ranging from 1uM and 10uM. Serial dilutions of compounds of the invention were dispensed into a 96-well plate (GREINER BIOSCIENCES) at 6 micro L each. Purified full-length human SYK, ZAP70 and truncated human RET and JAK2 (CARNA BIOSCIENCES) were diluted in kinase buffer and added to the compound solutions and pre-incubated for 30 minutes at room temperature (1 hour for PYK2). Next, ATP (TEKNOVA) and substrate solution (suggested manufacture substrates of PerkinElmer™, for example, Ulight™-TK peptide for SYK, Ulight™-PolyGT for ZAP70, FAK, and PYK2 (PERKINELMER)) was added (12uL each) to the wells containing the compound solution and enzyme. The reaction mixture was incubated for 1 hour (2 hours for PYK2). Following the incubation, the stop solution made with EDTA, water, and Lance detection buffer (PERKINELMER) was added (12 micro L each) to stop phosphorylation. Following the addition of the stop solution and 5 minutes of shaking, the detection solution containing the Europium-labeled antibody (suggested manufacture substrates of PerkinElmer™, for example, PT66 for SYK, ZAP70 and PYK), water, and Lance detection buffer was added (12mmicro L each) to the reaction mixture and incubated again for 50 minutes. Substrate phosphorylation was a function of the 665nm emission measured following the addition of the detection solution and 50 minutes of incubation. The IC_{50} value of test compound was calculated at Gradpad Prism 5 unless specified otherwise.

24. *Kinases with > 70% inhibition at 0.1 μM*: Abl(Q252H)(h), EGFR(T790M)(h), EGFR(T790M, L858R)(h), Flt3(D835Y)(h), Plk3(h), Ret(V804L)(h), Ret(V804M)(h); kinases with > 90% inhibition at 0.1 μM: MLK1(h); kinase with <25% inhibition at 0.1 μM: Fgr(h), Fyn(h), Lck(h), Lyn(h), Yes(h), Src(1-530)(h), Src(T341M)(h), Hck(h), Blk(h); kinases with <50% inhibition at 0.01 μM: Abl(Q252H)(h), EGFR(T790M)(h), EGFR(T790M, L858R)(h), Flt3(D835Y)(h), Plk3(h), Ret(V804L)(h), Ret(V804M)(h), MLK1(h).

25. *TNF-α release assay* : Compounds of the invention were tested for their TNF-α release effect on THP-1 cells. For SYK dependent TNF-α release assay (i.e., via IgG stimulation), THP-1 cells derived from human monocytic cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). This cell line was maintained with an Roswell Park Memorial Institute (RPMI) medium (GIBCO) containing 10 fetal bovine serum (FBS; GIBCO) and 0.05 mM 2-mercaptoethanol. The THP-1 cells were seeded at 1x 105 cells/100 micro L/well into human IgG (10 micro g/well, INVITROGEN)-coated 96 well culture plate, and serially diluted compound was then added. After an 18-hour incubation period at 37℃, supernatants were collected for the determination of the TNF-α level by enzyme-linked immunosorbent assay (ELISA), and the remaining cells were subjected to an MTT (yellow tetrazolium salt) assay to determine the cytotoxic effects of compound. For SYK independent TNF-α release assay (i.e., via lipopolysaccharide (LPS)-stimulation), THP-1 cells derived from human monocytic cells were obtained from the American Type Culture Collection

(ATCC, Manassas, VA). This cell line was maintained with an RPMI medium (GIBCOÔ) containing 10 fetal bovine serum (FBS, GIBCO) and 0.05 mM 2-mercaptoethanol. The THP-1 cells were seeded at 1x 105 cells/100 micro L /well into 96-well culture plates, and treated with lipopolysaccharide (1 micro g/ml), and serially diluted compound was then added. After an 18-hour incubation period at 37°C, supernatants were collected for the determination of the TNF-α level by ELISA, and the remaining cells were subjected to an MTT assay to determine the cytotoxic effects.

26. The structure was deposited in the Protein Data Bank ID code 4RSS. Binding mode was analyzed using program Ligand Explorer in PDB.

27. hERG testing was performed at Millipore IonChannelProfiler™ and ChanTest® Manual Patch Clamp assay.

28. Neither toxicity nor mutagenic activity has been observed at 5000 ug/plate in 4 Samonella typhimurium strains and 1 Escherichia coli strain (TA98, TA100, TA1535, TA1537 and WP2 uvrA ± liver S9 fraction).

29. The Collagen Induced Arthritis (CIA) mouse model was carried out at Oscotec Inc. (Republic of Korea). Male mice of DBA1/J strain were injected intradermally with bovine type II collagen and Complete Freund's Adjuvant at day 0 and 21 (boost at day 21). Mice develop inflammation 3 weeks after the first collagen injection. A chronic inflammation in the joints of the animals is achieved within 7–10 days after disease onset. Disease progression was monitored by scoring the severity of arthritis in four paws. Animals were euthanized on Day 42. The compound was administered orally continued once daily and twice daily through study.

Graphical Abstract

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Highly potent and selective pyrazolylpyrimidines as Syk kinase inhibitors

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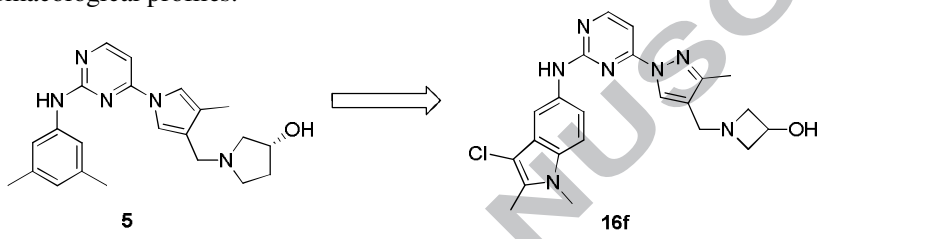
Jang-Sik Choi, Hae-jun Hwang, Se-Won Kim, Byung il Lee, Jaekyoo Lee, Ho-Juhn Song, Jong Sung Koh, Jung-Ho Kim*, Phil Ho Lee*

Indole substituted pyrazolylpyrimidine compound (**16f**) was identified as a potent and selective Syk inhibitor with good pharmacological profiles.



5

IC_{50} = 2.3 nM
Oral bioavailability (%F) = 7.6% (rat)

16f

IC_{50} = 1.0 nM
Oral bioavailability (%F) = 28.4% (rat), 46.4% (dog)